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Mitochondrial DNA deletion: A cause of chronic tubulointerstitial nephropathy

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Mitochondrial DNA deletion: A cause of chronic tubulointerstitial nephropathy. We report the first case of a mitochondrial DNA (mtDNA) deletion diagnosed by renal biopsy. An eight-year-old girl with megaloblastic anemia and severe growth retardation developed progressive renal insufficiency accompanied by partial Fanconi syndrome. Histologic examination of the renal biopsy disclosed nonspecific chronic tubulointerstitial disease characterized by tubular atrophy and interstitial fibrosis. On ultrastructural examination, tubular cell mitochondria were extremely dysmorphic with prominent size variation, abnormal arborization, disorientation of the cristae and osmiophilic electron-dense inclusions. Functional histochemical stains for mitochondrial enzymes performed on cryostat renal sections revealed focal tubular absence of cytochrome C oxidase (COX), a respiratory chain enzyme partially encoded by mtDNA, with preservation of succinate dehydrogenase (SDH), a respiratory chain enzyme entirely encoded by nuclear DNA (nDNA). Immunoreactivity for COX subunit 2 (encoded by mtDNA) was weak to undetectable in most tubular cells, whereas reactivity for subunit 4 (encoded by nDNA) was intense in all cells. Molecular analysis of the mtDNA of kidney and peripheral blood leukocytes was performed using Southern blot and PCR. Both techniques disclosed a 2.7 kb mtDNA deletion located between nucleotide (nt) 9700 and nt 13700, a common site for mtDNA deletions associated with encephalomyopathies. Mitochondrial DNA deletions may be an under-recognized cause of idiopathic tubulointerstitial nephropathy in children lacking neurologic or myopathic manifestations.

Mitochondria are the only subcellular organelles equipped with their own DNA (mtDNA). The 16.5 kb, circular, double stranded mtDNA encodes 13 structural proteins, all of which are members of the respiratory chain complexes; only complex II (succinate dehydrogenase) is entirely encoded by nuclear DNA (nDNA) [1, 2]. MtDNA also encodes 22 tRNAs and the 12s and 16s rRNAs comprising the small and large subunit of mitochondrial ribosomes, which are required for intramitochondrial protein synthesis. MtDNA is transcribed polycistronically and further enzymatically processed into mature messenger RNAs (mRNAs). At least one of these transcription factors (mtTF-1) [1] is encoded by nDNA. Furthermore, nDNA encodes most of the mitochondrial structural proteins as well as enzymes involved in mtDNA replication and repair [2, 3]. Obviously, dysfunction of such nDNA encoded proteins may cause defects of the mtDNA. Thus, mutations of nDNA, mtDNA, or both, may constitute the genetic basis of mitochondrial diseases.

Mitochondrial diseases have heterogeneous clinical phenotypes [4]. Organ systems most commonly involved are the central nervous system, skeletal muscles, cardiac conduction system, hematopoietic system and pancreas [5, 6], whereas liver, endocrine system and kidney [7–9] are affected infrequently and usually late in the course of the disease. MtDNA deletions have been found in patients with Pearson syndrome [10], a disorder manifesting in infancy and characterized by refractory sideroblastic anemia, thrombocytopenia, neutropenia, pancreatic insufficiency, renal tubulopathy and hepatic dysfunction accompanied by lactic acidosis [5, 8, 11]. Certain forms of mitochondrial encephalomyopathies are also associated with mtDNA deletions [12–14]. All of them exhibit progressive external ophthalmoplegia, either alone or as part of the multisystem Kearns-Sayre syndrome (KSS), which also includes pigmentary retinopathy and one of the following: heart-block, cerebellar dysfunction or elevated CSF protein level (>100 mg/dl) [2]. Renal manifestations are infrequent in KSS [15]. Our patient presented with anemia and renal disease and only later developed external ophthalmoplegia. Ultrastructural, histochemical, and immunohistochemical analysis of renal tissue obtained by percutaneus renal biopsy indicated a mitochondrial disorder, which was confirmed by molecular genetic analysis showing a mtDNA deletion.

Case report

This nine-year-old girl was the 2.9 kg product of a 37 week gestation, born to non-consanguineous parents with no family history of genetic disorders. Her 18-year-old brother is alive and well. Her pre- and peri-natal history were unremarkable. At the age of three years she developed megaloblastic anemia (Hct = 30%, Hb = 8.6 g/dl, MCV = 97 fl, Reticulocytes = 2.1%, WBC = 5.5 × 10^9/liter, Plt = 178 × 10^9/liter). Further laboratory work-up was undertaken and normal results were obtained for serum concentrations of: folate, bilirubin (direct, indirect, and total), LDH, uric acid, amylase, T3, T4, TSH, Fe, TIBC, ferritin, and free erythrocyte protoporphyrin. Serum B-12 concentration was found to be elevated at 1600 mg/dl (nl. = 200 to 950). Normal or negative results were also obtained from Hamm's test and sucrose gradient RBC incubation, hemoglobin

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electrophoresis, quantitative HbF, direct Coomb's test, antinuclear antibody, and HIV antibody. Studies of di-epoxy butane chromosome fragility gave normal results. Serum concentrations of BUN (=10 mg/dl), and creatinine (=0.5 mg/dl) were normal. Abdominal ultrasound demonstrated large kidneys and a very echogenic pancreas. A bone marrow aspirate displayed normal cellularity with a 3 to 1 ratio of myeloid to erythroid precursors and an adequate number of megakaryocytes. Cytoplasmic vacuolization of myeloid and erythroid precursors was noted. By the age of five years, her growth parameters dropped from the 50th to the 10th percentile for height and from the 10th to the 5th percentile for weight.

At the age of seven years, urinalysis disclosed 2+ proteinuria without formed elements. Serum creatinine was elevated at 0.9 mg/dl (estimated GFR = 71.5 ml/min./1.73 m^2). By the following year she had developed renal glycosuria (blood glucose = 97 mg/dl). A further rise of her serum creatinine to 1.6 mg/dl prompted referral to a pediatric nephrologist. At that time, other laboratory results were notable for a markedly elevated urine protein/urine creatinine ratio of 9.4. Serum albumin and cholesterol concentrations were normal. In addition, she had developed a metabolic acidosis (plasma HCO_3^- = 17 mmol/liter), hyperkalemia (plasma K^+ = 5.8 mmol/liter), hypophosphatemia (serum phosphate = 3.4 mg/dl) with a high fractional excretion of phosphate of 28% (expected value = 15 to 20% for a patient of comparable age and diet, with a normal serum phosphate concentration) and normal values for serum Ca^{++} (=9.1 mg/dl) and intact PTH (=36 pg/ml; nl. = 10 to 65 pg/ml). Alkaline phosphatase was mildly elevated at 408 U/liter. Urinary acidifying capacity seemed preserved with a urine pH of 5.5 at a time when plasma HCO_3^- was 21 mmol/liter. Calculation of the trans-tubular potassium gradient (TTKG = urinary K^+ × plasma osmolarity/plasma K^+ × urine osmolarity [16]) on two occasions gave values of 0.57 and 1.0. Concomitant respective values for fractional excretion of sodium of 10.5% and 13.6% documented adequate distal delivery of sodium. Rodriguez-Soriano, Ubetagoyena and Vallo [17] demonstrated that in children with adequate distal sodium delivery a TTKG value below 4.1 is consistent with the diagnosis of decreased aldosterone effect on distal tubular segments. Thus, in our patient the hyperkalemia was likely the result of decreased action of aldosterone on the distal and cortical collecting tubules. That this decreased action of aldosterone was due to tubular insensitivity to the hormone rather than to aldosterone deficiency was suggested by the measurement of a normal aldosterone concentration of 4.0 ng/dl (normal = 4 to 31 ng/dl) at a time when her serum potassium concentration was being maintained in a normal range by use of sodium polystyrene sulfonate and her blood pressure was being controlled with lisinopril. Urine output was maintained at 1000 to 1200 ml/day. Sodium bicarbonate (975 mg tid) and calcitriol (0.25 μg qod) were begun and successfully corrected the metabolic abnormalities resulting from her chronic renal insufficiency.

Due to the patient's progressive, non-oliguric renal insufficiency associated with heavy proteinuria and tubular defects in phosphate and glucose reabsorption, a percutaneous renal biopsy was performed. After the biopsy diagnosis, additional treatment was implemented (Coenzyme Q 60 mg tid, vitamin C 250 mg tid, folic acid 1 mg tid, Carnitine 500 mg tid, vitamin E 400 mg qd). In addition, an attempt was made to reduce renal tubular energy expenditure by adding NaCl 1 gm tid. The salt therapy had to be discontinued due to the development of edema and hypertension. No neurologic symptoms were evident at the time of renal biopsy. Over the ensuing year the patient developed decreased mobility of extraocular muscles and bilateral ptosis of the eyelids. Eye movements improved slightly after therapy was initiated. A 24-hour EKG recording was normal. Over the 20 months since the biopsy, her renal function has continued to deteriorate (most recent creatinine = 3.7 mg/dl) and she has developed hypertension requiring treatment with lisinopril and nifedipine.

Methods

The renal biopsy was divided in three pieces. The first was snap-frozen in 2-methyl butane (−80°C), the second was fixed in cacodylate buffered glutaraldehyde and processed for electronmicroscopy, and the third was fixed in Bouin's fixative and processed for routine histology.

Histochemical and immunohistochemical analysis was carried out on frozen renal tissue. A case of chronic tubulointerstitial nephritis served as control. Six-micrometer thick cryostat sections were stained for cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) by adding the corresponding substrates (cytochrome c and succinate) together with the chromogens diaminobenzidine (for COX) and nitroblue-tetrazolium (for SDH) [13]. COX (complex IV) is encoded by both mtDNA and nDNA; SDH (complex II) is encoded entirely by nDNA. To evaluate whether lack of COX function was due to absence of mtDNA or nDNA encoded portions of the complex IV (COX), frozen sections were stained with antibodies to subunits 2 (mtDNA) and 4 (nDNA) of COX using an indirect immunofluorescence technique [13].

Total DNA for Southern blot and PCR analysis was obtained from frozen renal tissue and peripheral blood leukocytes. Normal leukocytes were used as control.

Southern blot analysis

Five micrograms of total DNA were digested with PvuII. The restriction fragments were separated by electrophoresis on a 0.8% agarose gel and subsequently hybridized with a ^{32}P labeled probe composed of the entire mitochondrial genome [6]. Since PvuII cuts mtDNA at only one site, wild type mtDNA migrates as a single 16.5 kb band. When a deletion of mtDNA is present, a second band representing the smaller mutant mtDNA is observed. By comparing the migration of these fragments with fragments of known size run concurrently, the approximate size of the deleted DNA fragment can be calculated.

PCR analysis

Four different oligonucleotide primers were used to determine the approximate region of the mtDNA deletion within the mtDNA genome [18]. One set of primers, the forward primer (F) binding to nucleotides (nt) 8.274-8.305 and the backward primer (B) hybridizing to nt positions 13.720-13.692, flanks the 4.9 kb region which is frequently deleted in patients with Kearns-Sayre syndrome ("common deletion"). The other two primers hybridize to nucleotide positions 9.744-9.765 F and 12.653-12.629 B. The conditions for the PCR have been described elsewhere [18].

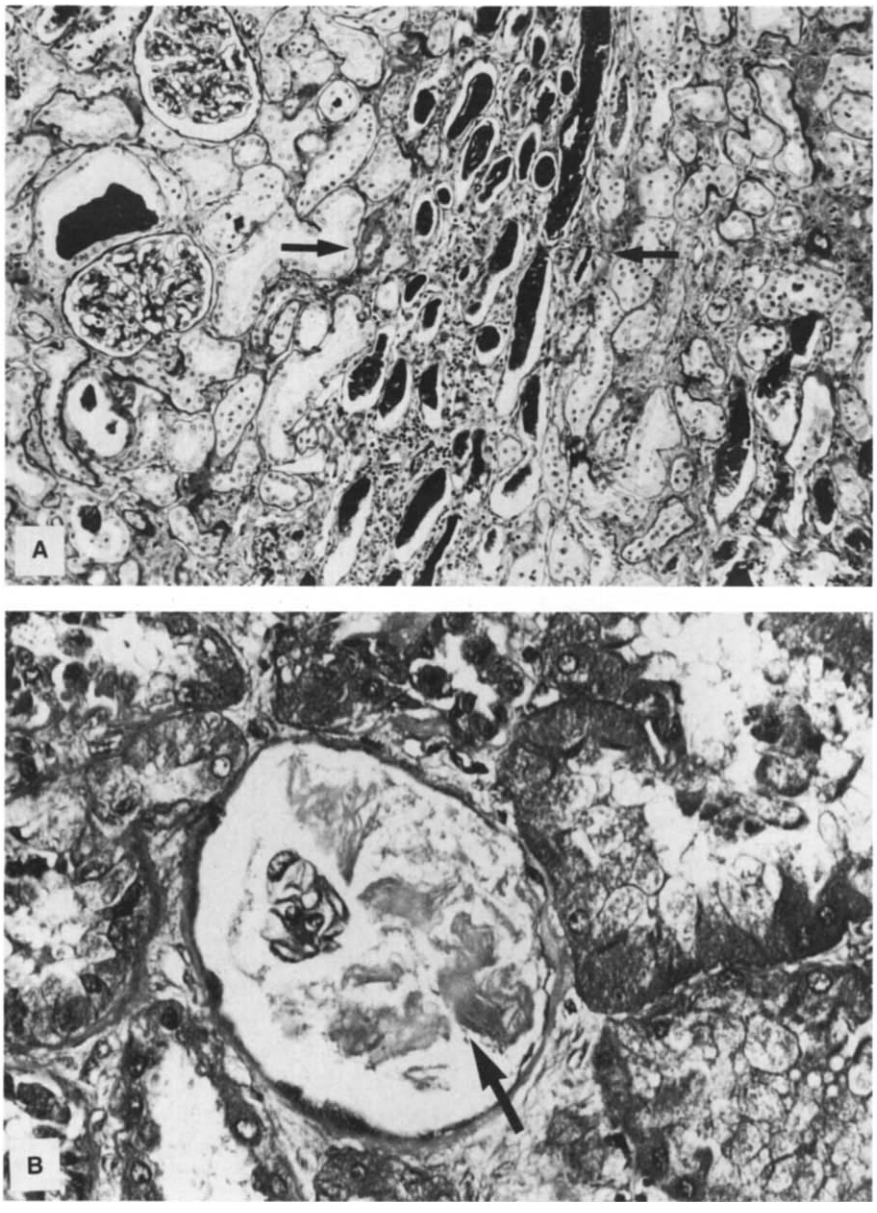

Fig. 1. *PAS stain (A) of renal biopsy showing stripe-like tubular atrophy of the medullary rays.* Most atrophic tubules contain PAS positive fibrillar casts (arrows) consistent with Tamm-Horsfall protein (THP) (×100). Masson's trichrome stain (B) demonstrates THP in Bowman's space (arrow) consistent with tubular reflux (×400).

Results

Histological findings

Approximately half of the glomeruli showed irregular retraction of the tuft with dilation of Bowman's space. There was chronic tubulointerstitial disease characterized by stripe-like moderate to severe tubular atrophy involving the medullary rays, associated with interstitial fibrosis (Fig. 1). Most of the atrophic tubules contained strongly PAS positive fibrillar casts suggestive of Tamm-Horsfall protein (Fig. 1). Similar casts were also observed in the medullary collecting tubules and infrequently in Bowman's space (Fig. 1). Routine immunofluorescence studies for immunoglobulin and complement were negative.

Ultrastructural findings

On ultrastructural examination proximal tubules displayed abnormalities in mitochondrial number, size and configuration (Fig. 2). Mitochondria were increased in number and showed prominent variation in size associated with abnormal arborization and disorientation of mitochondrial cristae including circular and parallel stack arrangements. Some mitochondria contained electron dense granular and fibrillar inclusions (Fig. 2). Other subcellular organelles and the tubular brush border appeared normal.

Histochemical and immunohistochemical findings

Atrophic tubules showed no reactivity for COX (brown reaction product), whereas SDH (blue reaction product) was

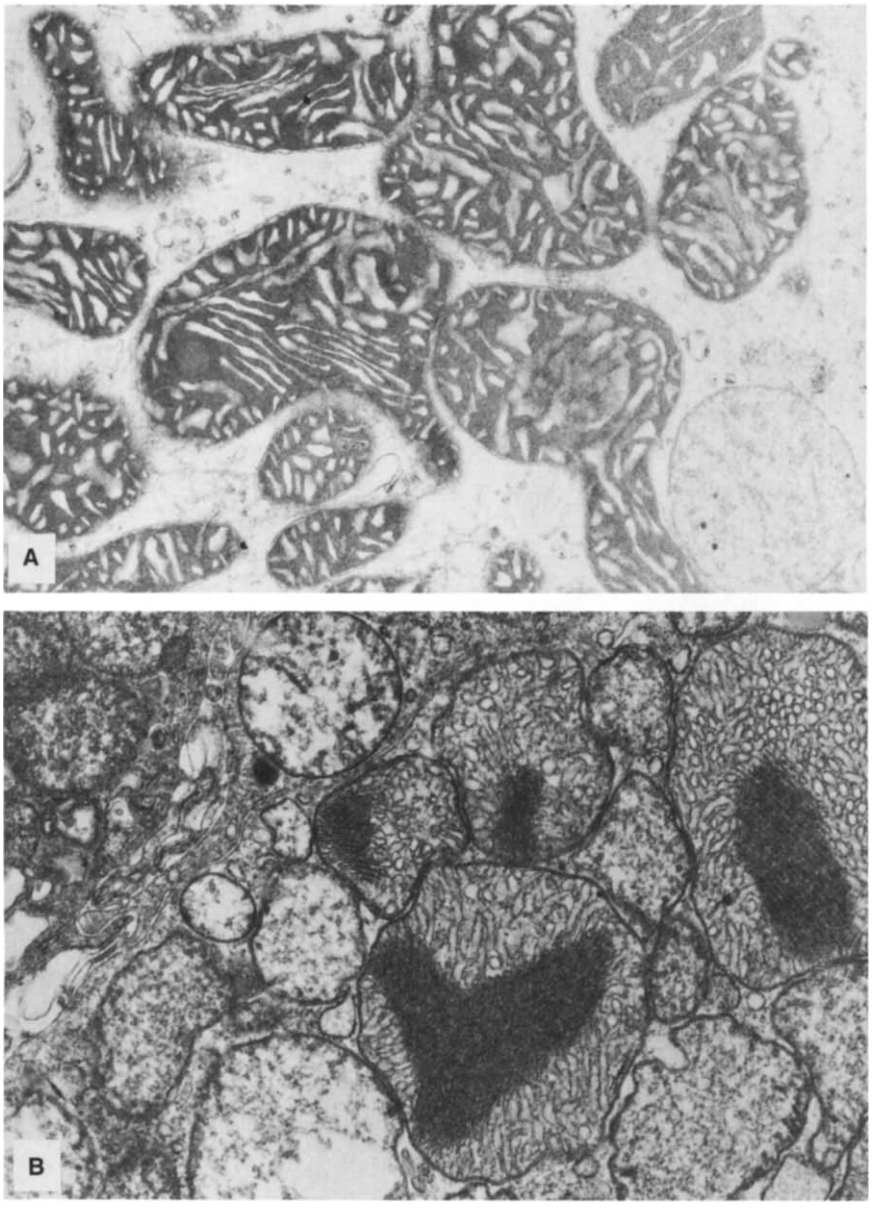

Fig. 2. *Electronmicrographs showing abnormal arborization and disorientation of mitochondrial cristae (A ×11700) and electron dense mitochondrial inclusions (B, ×10800).*

strongly reactive in all tubules (Fig. 3). The control sample from a patient with chronic drug-induced tubulointerstitial nephritis displayed diffuse positivity for both enzymes in atrophic and non-atrophic tubules. Since SDH is solely encoded by nDNA and COX is partially encoded by mtDNA (subunits 1 to 3), the selective absence of COX reactivity in our case pointed to a defect of the mtDNA. This assumption was corroborated by the fact that immunostaining was markedly reduced for the mtDNA encoded subunit 2 of COX (Fig. 4) and normal for the nDNA encoded subunit 4 of COX (Fig. 4). Control samples stained positive with both antisera.

Southern blot analysis

Southern blot hybridization performed on PvuII digested total DNA obtained from both renal tissue and peripheral blood leukocytes demonstrated an identical deletion of mtDNA. The 16.5 kb band, corresponding to the wild type mtDNA, was seen in all the control and patient samples. However, samples from the patient also displayed an additional band at approximately 13.7 kb, representing mtDNA with a deletion of 2.8 kb (Fig. 5). Peripheral blood leukocytes contained approximately 50% deleted mtDNA, whereas approximately 90% renal tubular cell mtDNA was deleted.

PCR analysis

Using PCR the approximate location of the 2.8 kb deletion was determined. Amplification with primers binding to nt 8.274-8.305 F and 13.720-13.692 B (Fig. 6, lane 1) and 9.744-9.765 F and 13.720-13.692 B (Fig. 6, lane 2) yielded PCR products of 2.7 kb and 1.3. kb, respectively, which are roughly 2.8 kb shorter than the expected sizes of 5.5 kb and 4.1 kb obtained for wild type mtDNA. The 5.5 kb and 4.1 kb band representing wild type mtDNA are absent in lanes 1 and 2, despite the presence of roughly 50% wild type DNA in blood leukocytes, because the PCR parameters (length of extension) chosen favored amplification of the shorter mtDNA fragment.

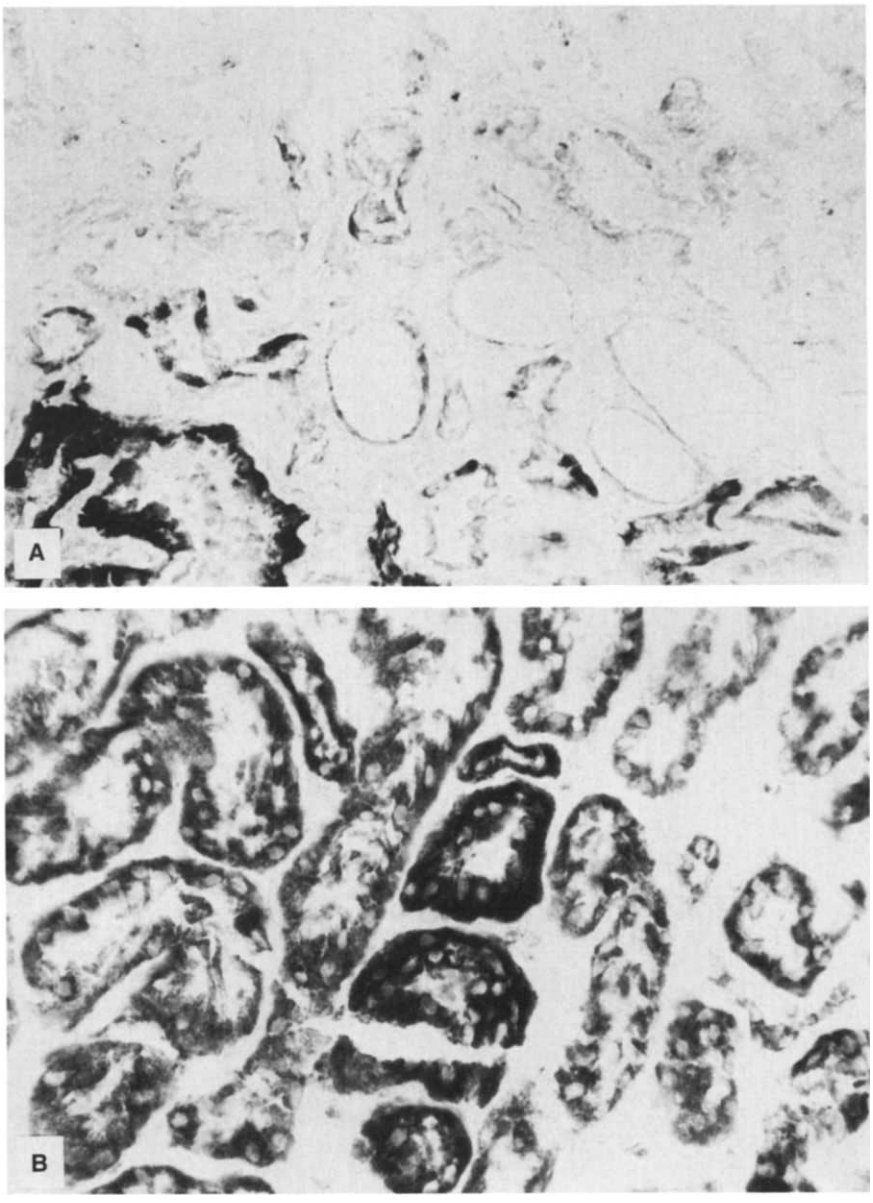

Fig. 3. *COX (A, B) and SDH (C, D) histochemistry of renal tubular cells.* COX reactivity of the patient's biopsy (A) is markedly reduced compared to the control (B). SDH staining appears equally intense for the patient's biopsy (C) and the control specimen (D) (×400).

The deleted segment was localized between nucleotide position 9.765 and 13.692, because the primers 9.744-9.765 F and 13.720-13.692 B (Fig. 6, lane 3) hybridized to the mutated mtDNA, whereas PCR amplification using primer 12.653-12.629 B showed only amplification of wild type mtDNA represented by the 4.4 kb band in lane 3 of Figure 6. The absence of PCR product from mutated mtDNA using primer 12.653-12.629 B indicates that this mtDNA segment is part of the 2.8 kb deletion.

Discussion

This case represents the first example of a mtDNA deletion diagnosed by renal biopsy. The patient reported herein had been followed for refractory megaloblastic anemia and growth retardation for several years before the development of partial Fanconi syndrome and progressive deterioration of renal function. Mild external ophthalmoplegia with ptosis of the eyelids occurred only after the diagnosis of a mitochondrial disorder associated with mtDNA deletion had been made by percutaneous renal biopsy. Refractory anemia and progressive external ophthalmoplegia are features of Pearson syndrome and Kearns-Sayre syndrome (KSS), respectively. MtDNA deletions are invariably present in both syndromes [5, 6]. As in our patient, features of each of these clinical syndromes may co-develop in the same patient [19]. Reportedly, survivors of Pearson syndrome may develop KSS later in life. Although Pearson syndrome is frequently associated with renal involvement [8, 11, 19], the clinical picture of our patient is unusual for the severe degree of renal insufficiency, whereas the anemia was comparatively mild. It was this disproportionately severe renal involvement that caused her to come to the attention of a nephrologist, and ultimately led to the renal biopsy diagnosis of a mtDNA deletion.

The wide spectrum of clinical manifestations of mitochondrial disorders is related to specific features of mtDNA genetics which are different from nDNA genetics [2, 20]. Most cells,

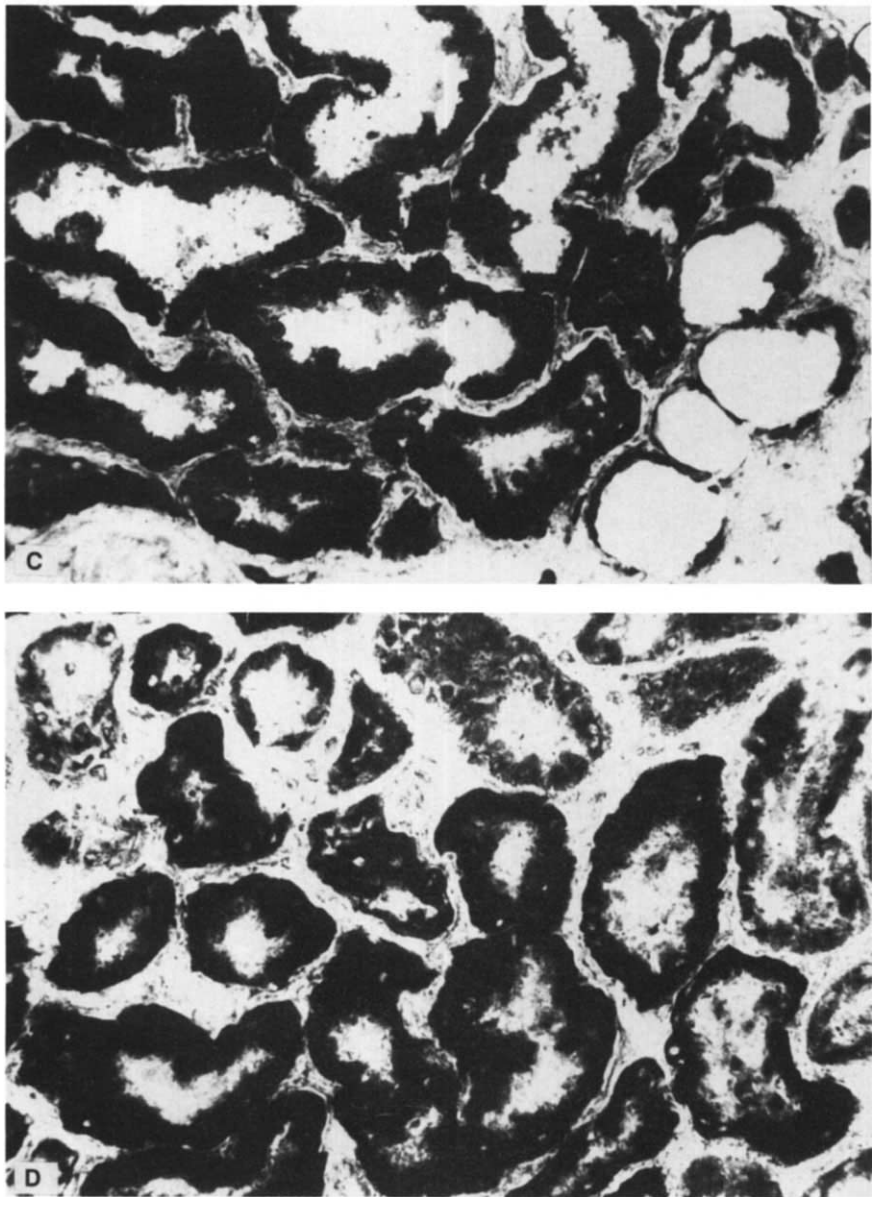

Fig. 3. *Continued*

including the oocytes, contain thousands of copies of mtDNA with approximately 10 to 12 copies of mtDNA present in each mitochondrion. Only a fraction of mtDNA carries the respective mutations. This leads to coexistence of mutated and wild type mtDNA in the same cell, a phenomenon known as heteroplasmy [2, 21]. Since all mitochondria derive from the oocyte, mtDNA mutations are usually maternally inherited, an inheritance pattern different from Mendelian genetics [20]. Mendelian rules, however, are applicable to mitochondrial diseases caused by deficiencies of nDNA encoded mitochondrial proteins. MtDNA deletions are usually sporadic, as in our case. Supposedly, the event leading to mtDNA deletion occurs early in development, because mtDNA deletions co-exist in many organ systems. Only selected organ systems, however, show clinically significant dysfunction [4, 21]. Since mutated mtDNA is randomly distributed to daughter cells during mitosis, different cells may contain varying ratios of mutated versus wild type mtDNA (for example, heteroplasmy), explaining the clinical heterogeneity [15]. Two factors related to heteroplasmy account for clinically evident organ dysfunction. First, a threshold number of mutant mtDNA must be present for the disease to be expressed in a given tissue. Second, the threshold may vary in different tissues according to the dependence of each tissue on oxidative metabolism [2].

Our patient exhibited approximately 90% deleted mtDNA in renal tubular cells, whereas blood leukocytes contained approximately equal ratios of deleted and wild type DNA, thereby accounting for the dominance of renal symptoms. Such a large percentage of mutated mitochondria in renal tissue has not been reported previously. Interestingly, the structure most severely affected in our case was the medullary ray, which comprises the straight portions (predominantly S3 segment) of the proximal tubules as well as the cortical collecting tubules and the thick ascending limb of Henle. This was evidenced by the stripe-like

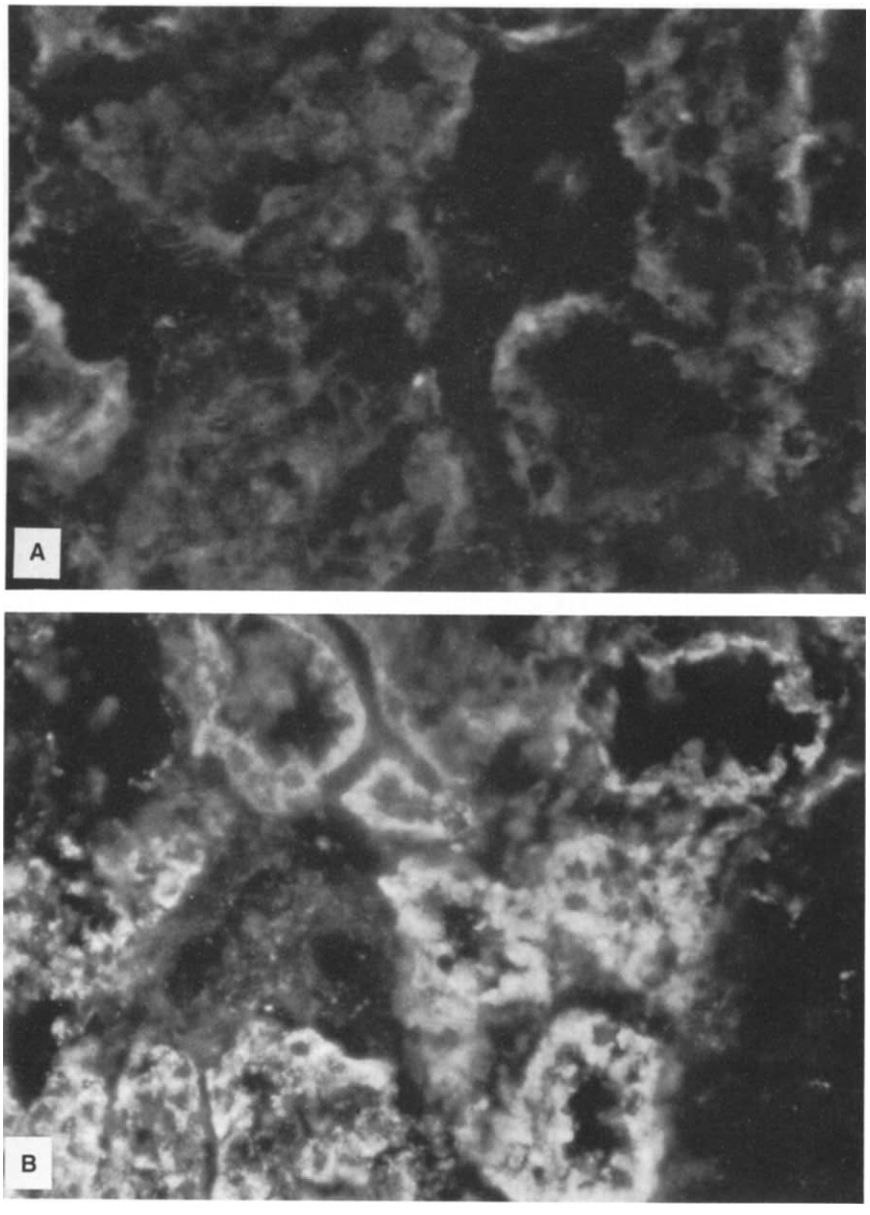

Fig. 4. *Immunofluorescence staining for COX subunits 2 (A) and 4 (B).* Renal tubular epithelial cells stain faintly for mtDNA encoded COX subunit 2, whereas staining for nDNA encoded subunit 4 is strongly reactive. The faint reactivity for COX subunit 2 in the patient's renal tubular cells is due to the presence of small amounts of wild type mtDNA. (×400).

fibrosis and presence of numerous casts containing Tamm-Horsfall protein. This portion of the nephron is particularly vulnerable to limited oxygen availability [22]. Similar histologic findings are observed with chronic cyclosporin toxicity [23] which is associated with renal ischemia [22].

It has been suggested that tissues with a high mitotic rate, such as hematopoietic cells, are able to select against heteroplasmic cells in the course of continued cell division [19, 21]. This phenomenon can lead to spontaneous resolution of anemia in older patients with long-standing Pearson syndrome. Conversely, the ratio of deleted mitochondrial genomes increases with age in permanent tissues such as muscle and brain accounting for the usually late onset of progressive neuromuscular symptoms [24]. Since renal tubular cells have a high regenerative potential, renal symptoms may only be present in the early stages of mitochondrial diseases and resolve spontaneously. Therefore, clinical renal involvement in mitochondrial

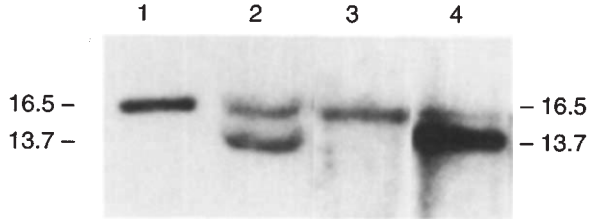

Fig. 5. *Southern blot of total DNA extracted from normal control leukocytes (lane 1) and control renal tubular cells (lane 3), the patient's leukocytes (lane 2) and patient's kidney (lane 4).* Five micrograms of DNA were digested with PvuII and hybridized with P^{32} labeled mtDNA. Samples of the patient (lanes 2 and 4) disclosed an additional band at 13.7 kb representing the deleted mtDNA.

disorders may be under-recognized in patients coming to clinical attention late in the course of their disease. In our case the severity of renal involvement necessitated a renal biopsy and

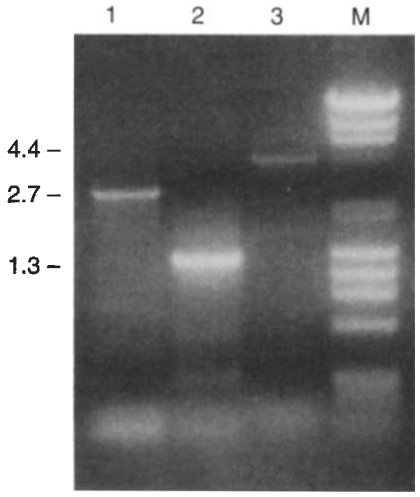

Fig. 6. *PCR of total DNA from patient's leukocytes using primers 8274-8305 F and 13720-13692 B (lane 1), 9744-9765 F and 13720-13692 B (lane 2) and 8274-8305 F and 12653-12629 B (lane 3).* The size of the PCR products was determined by markers of known length (lane M). Lanes 1 and 2 show bands of mtDNA, which are approximately 2.8 kb shorter than expected (2.7 kb instead of 5.5 kb in lane 1, 1.3 kb instead of 4.1 kb in lane 2), indicating that a 2.8 kb mtDNA deletion is located between nt 9765 and 13692. Lane 3 displays only the expected size (4.4 kb) of the wild type mtDNA fragment indicating that deleted mtDNA was not amplified, because there by the binding site of primer 12653-12629 B lies within the deleted segment, thereby providing a rough estimate of the location of the deletion within the mtDNA genome.

the mitochondrial abnormality could be documented by ultrastructural examination. Changes characteristic for mitochondrial disorders, particularly inclusions, were observed only focally, whereas more nonspecific changes including circular orientation of the cristae and aberrant arborization were more widely distributed [25]. Similar changes may also be observed with some forms of drug toxicity such as AZT [26]. In our patient the possibility of a primary mitochondrial disorder was only entertained after careful ultrastructural examination of the renal tubules revealed these mitochondrial abnormalities. In general greater attention is directed to the glomeruli in ultrastructural work-up of diagnostic renal biopsies, and detailed examination of the tubules is rarely done. More careful ultrastructural study of renal tubules may uncover a greater number of mitochondrial disorders among patients with idiopathic chronic tubulointerstitial nephropathy [9, 15, 27, 28].

The 2.8 kb mtDNA deletion observed in our patient is one of the shortest reported so far and does not involve any of the structural genes encoding subunits of COX. Nevertheless, histochemical and immunohistochemical stains revealed a widespread absence of functional COX in many renal tubules. This may be explained by the fact that this 2.3 kb deletion encompasses several tRNA genes, thereby affecting overall mitochondrial translation and leading to a general deficiency of all mtDNA encoded proteins [12]. This observation further emphasizes that the clinical phenotype is primarily determined by the tissue distribution and proportion of deleted mitochondrial genomes whereas the size and genomic location of the deletion appear to be less critical [17].

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